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14. ABSTRACT

BRCA1, or Breast Cancer Associated gene 1, is a breast and ovarian cancer-specific tumor suppressor. Recent research has shown that BRCA1, an E3 ubiquitin ligase, interacts in a complex that contains the human homologue of Ovarian Tumor (hOTU). Located within the OTU gene product is ~130 residue domain termed the Ovarian Tumor (OTU) domain that possesses deubiquitination activity.

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INTRODUCTION

BRCA1, or Breast Cancer Associated gene 1, is a breast and ovarian cancer-specific tumor suppressor. Recent research has shown that BRCA1, an E3 ubiquitin ligase, interacts in a complex that contains the human homologue of Ovarian Tumor (hOTU). Located within the OTU gene product is ~130 residue domain termed the Ovarian Tumor (OTU) domain that possesses deubiquitination activity. In this final report, I describe the synthesis of a covalent complex between the yeast OTU domain and ubiquitin, the purification, and crystal structure solved to 1.5 Ångstroms. I also report the *in vitro* characterization of biochemical activity of YOD1. Finally, I will describe the *in vivo* binding partners of YOD1 that have been identified. All tasks described in the Statement of Work have been completed.

Background

Women who have a germline loss-of-function allele of *BRCA1* are 85% likely to develop breast cancer and 63% likely to develop ovarian cancer by age 70.[1] In most familial cases of breast and ovarian cancers, germline mutations are followed by somatic loss or inactivation of the wild-type allele. We do not fully understand the exact mechanism by which functional loss of the BRCA1 protein leads to the development of cancer. Yet increasing evidence suggests that BRCA1 exerts its tumor suppressor functions, at least in part, by participating in certain DNA damage responsive pathways.

BRCA1 is a large, 1863-amino acid protein containing two identified motifs: two BRCT repeats at the C-terminus and a zinc-binding RING domain at the N-terminus. The BRCT (BRCA1 C-Terminal) repeats can form heteromultimers with other BRCT-containing DNA repair proteins, such as BACH1, a DEAH helicase. The N-terminal RING domain exhibits ubiquitin ligase (E3) activity. Many mutations within the RING domain, which result in loss of ubiquitin ligase activity, predispose patients for cancer.[2]

Ubiquitin is one of the most conserved proteins in eukaryotes. Protein ubiquitination—the covalent attachment of ubiquitin—controls a number of diverse biological processes including cell-cycle progression, signal transduction, receptor endocytosis, and fate determination. Ubiquitination of a protein substrate is carried about by three classes of enzymes: E1, in the presence of ATP, forms a high-energy thiol ester bond with ubiquitin, which is transferred to E2, a ubiquitin-conjugating enzyme. Finally, ubiquitin is transferred to a target substrate protein through an isopeptide linkage between the conserved C-terminal glycine residue and the ε-amino group of the lysine.

Ramin Shiekhatter's laboratory, at the Wistar Institute, has identified a number of BRCA1-interacting proteins. Using a FLAG-tagged RING domain segment of BARD1, they were able to identify the human homologue of *Drosophila* Ovarian Tumor, or hOTU. They also discovered that another BRCA-interacting protein CtIP interacts with hOTU. This interaction between the BRCA-containing complex and hOTU is strong; hOTU still remains bound to immunoprecipitated Flag-tagged BARD1 and CtIP during 500 mM KCl washes.

Located within the *Drosophila Otu* gene is a ~130 residue domain that is highly-conserved from human to yeast.[3] This region, termed the ovarian tumor (OTU) domain,

displays weak similarity to the papain-like family of cysteine proteases. The OTU domain is found in a number of conserved genes, from all eukaryotes and a number of eukaryotic viruses. In the yeast *Sacchromyces cerevisiae*, two ORFs (YFL044c and YHL013c) contain the OTU domain that we have named *YOD1* (Yeast OTU Deubiquitinating enzyme 1) and *YOD2*.

In order to understand the molecular mechanism and substrate specificity of this new class of deubiquitinating enzymes, I have concentrated this year on determining the three-dimensional structure of the catalytic domain of YOD1 in complex with ubiquitin.

Objectives: The objectives of these studies are three-fold:

(1) Determine the three-dimensional structures of an OTU-domain containing protein alone and in complex with ubiquitin substrate.

We aim to elucidate the structural basis by which OTU domains remove ubiquitin from protein-ubiquitin substrates. To that end, I propose to determine the three-dimensional structure of an OTU domain in isolation and in a complex with a ubiquitin substrate. There exists a relationship between structure and molecular function. Having the structure in-hand will allow us to address questions of substrate specificity and the molecular mechanism of OTU-containing enzymes.

(2) Characterize *in vitro* the biochemical properties of YOD1 using a deubiquitination assay. For any newly discovered deubiquitinating enzyme, the identification of the level of biochemical activity is an important priority. Incubating an OTU-containing enzyme with ubiquitin-7-amido-4-methylcoumarin (Ub-AMC), an ubiquitin derivitized at the C-terminus with AMC, allows one to monitor the reaction when AMC, a highly fluorogenic marker, is released by catalysis. This *in vitro* assay will allow the determination of the dissociation rate, K_d, the rate of the reaction, k_{cat}, and lead to an increased understanding of the reaction mechanism for this new family of deubiquitination enzymes. The assay will also permit us to confirm hypotheses generated by the three-dimensional structure concerning the mechanism and substrate specificity of OTU domains.

(3) Determine the *in vivo* ubiquitin-protein substrates of YOD1 proteins using a substrate-trapping method and two-hybrid system. The determination of the *in vivo* substrates will allow the identification of the pathway in which OTU domain-containing enzymes are participating.

BODY

Task 1—Crystallography and Structure Determination

During the funding period, all tasks related to Aim 1 (Task 1 A-C) have been completed. As outlined below, I have successfully expressed and purified an irreversible ubiquitin inhibitor. I created a covalent complex between ubiquitin and the yeast OTU domain, and purified the complex. Two crystal forms of the OTU-ubiquitin complex were obtained. Two Multiple Anomalous Dispersion (MAD) datasets and a native dataset, which diffracted to 1.5 Ångstroms, were collected at the National Synchrotron

Light Source (NSLS). More detailed information about the methodology and the results of the experiments follows.

Methods

Eleven constructs of OTU domain-containing proteins from different species were created. YOD1 (amino acids 87-262) was cloned into the pTYB2 vector (New England Biolabs). *E. coli* harboring the pTYB2-YOD1 (87-262) vector were grown, induced and lysed in a Tris pH 8.0 buffer containing 1 M NaCl and protease inhibitors. Lysate was applied to a chitin column, and washed extensively with 50 mM Tris 8.0 and 1 M NaCl. After washing, cleavage of the intein-chitin-binding domain (CBD) fusion was achieved by incubating with 50 mM DTT in the wash buffer overnight and room temperature. Milligram quantities of soluble YOD1 were then concentrated using an Amicon filtration unit (10 K MWCO), and applied to an Amersham-Pharmacia Superdex 75 column that was equilibrated with PBS pH 7.4. Seleno-methionine substituted YOD1 was expressed in the methionine-auxotroph B834 strain in methionine-free media containing 50 μM seleno-methionine, and purified and concentrated as above.

The ubiquitin gene (amino acids 1-75) was also cloned into pTYB2 vector. Ubiquitin(1-75)-intein-CBD were also expressed and purified as with the YOD1 preparation. However, the elution of ubiquitin was achieved using 50 mM β-mercapto-ethansulfonic acid (Mes Na) overnight at 37˚ C in a buffer containing 50 mM Cacodylate pH 6.5 and 100 mM sodium acetate. The eluted protein was concentrated to 8-12 mg/ml and dialyzed overnight in 50 mM cacodylate pH 6.5 and 100 mM sodium acetate using Pierce slide-a-lyzers 5000 MWCO. To create ubiquitin-Br3, 50 mM 3-bromopropylamine was added and the mixture was incubated for 2 hours at 37˚ C. After incubation, the sample was then dialyzed in PBS pH 7.4 using the Pierce slide-a-lyzers overnight at room temperature.[4]

To create the complex between YOD1 (87-262) and ubiquitin-Br3, equal molar amounts of each were incubated at 37˚ C for 2 hours. The complex was then purified using a High-Q EconoPac column from BioRad and gradient eluted from the column ~100 mM NaCl. Fractions were then concentrated to ~20 mg/ml and prepared for crystallization.

Two crystal forms were obtained from two different conditions. Crystals of the hexagonal crystal form were grown by the hanging-drop method by mixing the complex with an equal volume of reservoir solution containing Bis-Tris pH 5.5-6.5, 16-20% PEG 3350, and 100-200 mM of a magnesium cation. These crystals belong to spacegroup $P6_4$ with unit cell dimensions of 107.3 x 107.3 x 100.2 Å $\alpha=\beta=90^\circ$ $\gamma=120^\circ$ with 2 molecules in the asymmetric unit. Orthorhombic crystals were also grown by hanging-drop method using a reservoir solution containing Bis-Tris pH 5.5-6.5, 16-20% PEG 3350, and 50-200 mM ammonium acetate . Orthorhombic crystals belong to spacegroup $P2_12_12_1$ with unit cell dimensions 46.2 x 73.2 x 88.8 Å $\alpha=\beta=\gamma=90^\circ$. The orthorhomic crystal form was solved by soaking in platinum into the crystals before cryofreezing. The hexagonal crystal form was solved using a selenium substituted methionine (Se-Met) protein. Data was collected and processed at X29 at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL) in Upton, NY.

$P2_12_12_1$	Peak	λ=1.0722	Inflection	λ=1.0725	Remote	λ=1.0450	Native	λ=0.9795
Resolution	20.0-2.0	2.07-2.00	20.0-2.0	2.07-2.00	20.0-2.0	2.07-2.00	10.0-1.48	1.53-1.48
Completeness	99.9	100	99.9	99.9	99.9	100	98.3	93.7
I/σ	16.84	13.81	19.92	5.06	25.80	9.11	26.74	3.39
R_{sym}	0.095	0.164	0.094	0.381	0.073	0.228	0.048	0.478

$P6_4$	Peak	λ=0.9793	Inflection	λ=0.9795	Remote	λ=0.9566
Resolution	50.0-2.31	2.39-2.31	50.0-2.31	2.39-2.31	50.0-2.40	2.49-2.40
Completeness	99.5	95.7	98.3	89.4	95.9	80.2
I/σ	23.30	6.23	26.62	3.29	21.11	1.69
R_{sym}	0.126	0.445	0.090	0.611	0.124	0.863

Table 1. Data processing statistics for two crystal forms of YOD1-Ubiquitin complex.

Data processing statistics for the two crystal forms of YOD1 complexed with ubiquitin are described in Table 1. Data was processed using HKL2000.[5] For the orthorhombic crystal form, three-hundred-sixty 1˚ frames were collected for each of the three wavelengths. One-hundred fifty degrees of data was collected for the native wavelength. For the hexagonal crystals, one-hundred-twenty 1˚ frames were collected for the direct and the inverse beams for each of the three wavelengths.

The structure was determined by multiple anomalous dispersion (MAD). Using a combination of SOLVE/RESOLVE[6] and SHARP, 8 selenium sites and 6 platinum sites were identified. For the orthorhomic crystal form, initial MAD phases were extended to 2.0 Ångstroms and improved with solvent flattening and histogram matching. A model was easily built into the electron density using ARP/wARP[7]. This model was then used for refinement against the native 1.5 Å dataset. For the hexagonal crystal form, two molecules in the assymetric unit were identified. Initial MAD phases were also solvent flattened, and a model was unambiguously traced.

Results

YOD1 (amino acids 87-262) was expressed in *E. coli* and purified to homogeneity. Ubiquitin (1-75) was also expressed in bacteria and cleaved off the chitin column using MES Na. The synthesis of an irreversible ubiquitin inhibitor (ubiquitin-Br3) was achieved by chemically ligating 3-aminopropylbromide on to the C-terminus of ubiquitin-MES Na. A covalent complex was created by incubating equal molar amounts

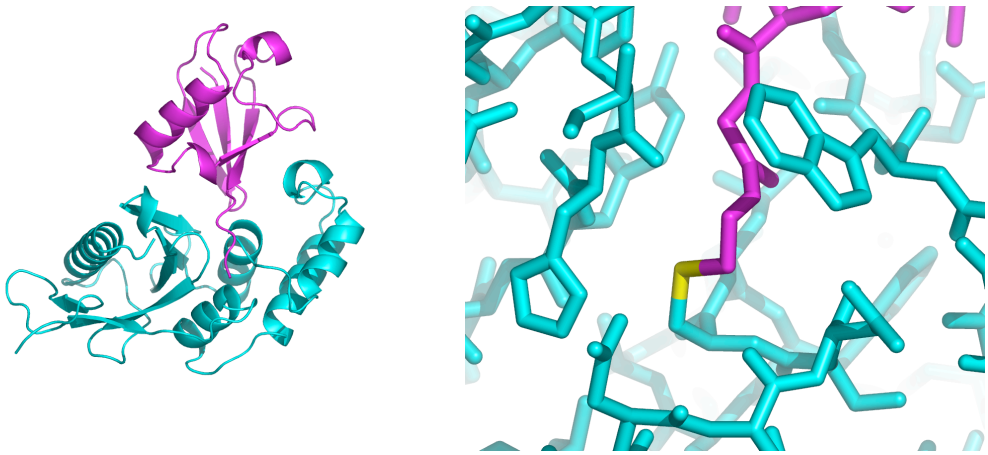

Figure 1. Left, ribbon diagram of ubiquitin in magenta bound to the YOD1 (87-262) in cyan. Right, the active site of the OTU domain. The C-terminus of ubiquitin in magenta is bound covalently to the YOD1 in cyan via a thiolester linkage in yellow from the cysteine 120 of YOD1.

of YOD1 and ubiquitin-Br3. Approximately 50% of the YOD1 and 50% of the ubiquitin-Br3 reacted to create the complex. The complex was purified from unreacted protein using a Q column.

The OTU catalytic core domain resembles a hand where the thumb contacts the backside of ubiquitin, the palm contains the active site, and the fingers contact make several contacts with the front side of ubiquitin (See figure 1). The size and shape of the OTU hand appear to be ideal for binding the 8.5 kDa ubiquitin molecule. The thumb comprises two alpha-helices culminating in a short alpha helix that extend from the palm to make several hydrophobic contacts with hydrophobic backside of ubiquitin. In particular, Alanine 156 of YOD1 interacts with Isoleucine 44 of ubiquitin. This residue has been utilized by a number of ubiquitin binding proteins to make contacts with ubiquitin.[8] The active site of YOD1 is made up of a bipartite pattern of conserved residues around Asp-X-X-Cys and a remote histidine. These three residues form a catalytic triad that prime the cysteine for nucleophilic attack on the gly-gly terminus of ubiquitin. Tryptophan 175 also serves to sandwich in the gly-gly motif in the active site (See figure 1). The finger portion of the OTU domain is composed of a core of six β-strands arranged in an anti-parallel fashion which surround one long alpha helix.

The structure of an OTU domain not bound to ubiquitin[9] reveals an extended loop that occludes ubiquitin from the active site. It appears that upon binding to ubiquitin, this loop adopts a beta strand fold and packs tightly against a number of charged residues on the surface of ubiquitin. (See figure 2.)

The analysis of the structure of YOD1 catalytic domain bound to ubiquitin is still ongoing. It is hoped that once the structure is fully refined, that a more careful analysis can be done to determine how the OTU domain recognizes and cleaves ubiquitin chains.

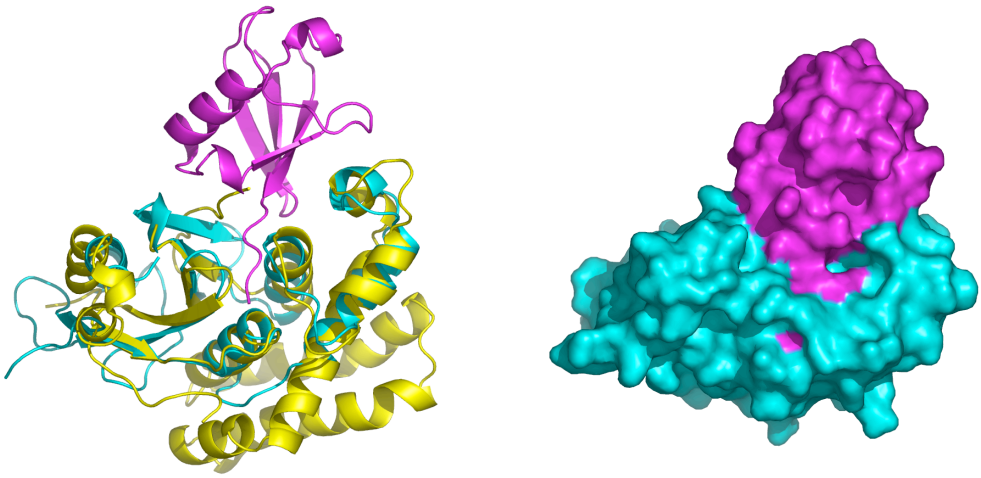

Figure 2. Left, the OTU domain in cyan bound to ubiquitin in magenta superimposed on the structure of an OTU domain unbound in yellow. A loop in the unbound structure adopts a β-sheet conformation upon binding to ubiquitin. Right, a surface representation of the OTU domain bound to ubiquitin.

Task 2—Biochemical characterization

During the funding period, all tasks related to Aim 2 (Task 2 A-C) have been achieved. I determined the k_{cat} and K_m of YOD1 (Task 2A). We mutated key residues (Task 2B) and expressed and purified the resulting mutants and determined their activity (Task 2C).

Methods and Results

To determine the functionality of our recombinant protein, I labeled recombinant full-length YOD1 with ubiquitin-vinyl sulfone (UbVS), an active site-directed irreversible inhibitor that reacts specifically with the active site cysteine of DUBs. Titration of 100 ng of YOD1 with increasing amounts of UbVS clearly shows the formation of a covalent adduct migrating about 10 kDa higher than YOD1 alone on SDS-PAGE (Fig. 3A). Significantly, the quantitative UbVS labeling of YOD1 indicates that almost all of the recombinant YOD1 is enzymatically competent. UbVS also completely inhibits YOD1 cleavage of Ub-AMC in fluorometric assays, indicating that the UbVS binds to the YOD1 active site.

I next examined the specific activity of YOD1. Previous data suggested that YOD1 was a very sluggish DUB, with an estimated velocity of ~0.01 s^{-1} at low substrate concentration. This slow activity might be due to the N-terminal ubiquitin foldlike (UBX) domain acting as an inhibitor by occupying the ubiquitin binding site of the enzyme. To test this hypothesis, recombinant full-length YOD1 and a truncated version missing an 86-residue N-terminal UBX domain, herein called YOD1ΔUBX, were incubated with increasing concentrations of the generic DUB substrate, Ub-AMC. Cleavage of Ub-AMC was monitored by the release of the fluorescent AMC molecule (Fig. 3B). These experiments reveal comparable kinetic behavior for both proteins with no evidence of saturation below 2 μM Ub-AMC and an apparent k_{cat}/K_m of 1×10^3 M^{-1} s^{-1}. This result shows that the UBX fold does not participate in catalysis by YOD1.

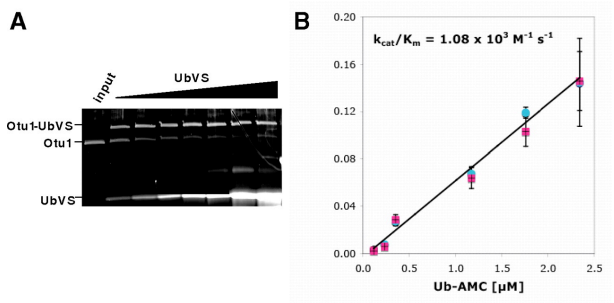

Figure 3. Kinetic analysis of YOD1. A, YOD1 labeling by ubiquitin vinyl sulfone. The quantitative labeling of YOD1 by ubiquitin vinyl sulfone was analyzed by SDS-polyacrylamide gel and stained with Sypro Ruby Red. B, deubiquitinase activity of YOD1. Lineweaver-Burk plot of activity data obtained for recombinant full-length YOD1 (blue diamonds) and YOD1ΔUBX (magenta boxes) using a ubiquitin-AMC substrate. The release of AMC as a function of added protein is monitored by fluorescence.

To establish the functional importance of the observed YOD1-ubiquitin interactions, I carried out a mutational analysis. For the mutagenesis, I targeted selected residues within contact regions 1-3 of YOD1 as well as the active site residues. Each mutant was prepared by site-directed mutagenesis in the context of the recombinant YOD1 domain used for structural analysis, purified to homogeneity, and assayed for deubiquitinating enzyme activity using the fluorescence assay described earlier with Ub-AMC as the substrate (Fig. 3B). The results of this assay are summarized in Fig. 4A. As a base line for these experiments, I mutated the catalytic triad residues (C120A, H222A, and D224A), and as expected, the C120A and H222A residues showed background levels of catalytic activity. However, the D224A mutant still showed about 30% of wild-type activity, suggesting that it plays a less critical role in catalysis consistent with its poorer conservation relative to the cysteine and histidine residues of the catalytic triad.

Within region 1 of the YOD1-ubiquitin interface, we mutated alanine 156 and isoleucine 157 to glutamate residues. Although the A156E mutation showed 50% of wild-type activity, the I157E mutation showed less than 10% of wild-type activity (Fig. 4A). Both residues mediate van der Waals contacts to a hydrophobic patch on ubiquitin that is centered around Ile44, a residue that appears to be widely recognized by ubiquitin binding domains, such as UIM, CUE, and GAT domains among many others. The conservation of residues Ala156 and Ile157 also points to their functional importance.

Within region 2 of the YOD1-ubiquitin interface, we mutated glutamate 207, a residue that appeared from the structure to mediate important direct and water-mediated interactions with Lys11 and Thr9 of ubiquitin. Surprisingly, the E207K mutant showed

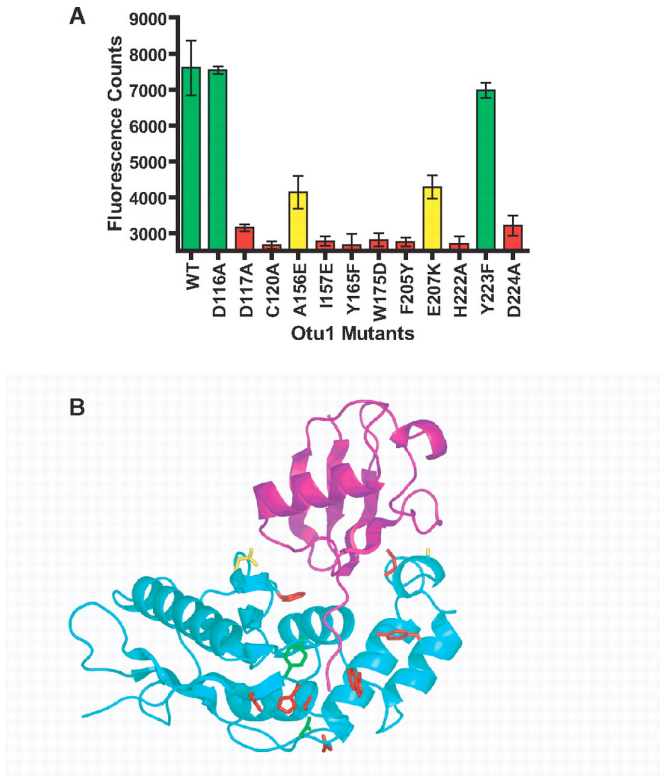

Figure 4. Mutational analysis of the YOD1-ubiquitin interface. A, deubiquitinase activity of recombinant YOD1 and single site mutants. Each of the reactions was performed in triplicate and in the linear range of time and enzyme and substrate concentration. Activities are color-coded according to severity (green, wild type activity; yellow, about 50% of wild-type activity; red, less than 30% of wild-type activity). B, mapping of mutational studies onto the YOD1-ubiquitin complex. Side chains that were mutated in A are color-coded according to their severity on enzymatic activity as described in A.

50% of wild-type activity, thus arguing against the relative importance of region 2 for YOD1-ubiquitin complex formation.

The structure of the YOD1-ubiquitin complex suggests that region 3 plays a particularly important role in complex formation, and this is supported by the mutational analysis. Specifically, three of the four mutations that we prepared in this region (Y165F, W175D, and F205Y) were significantly compromised in deubiquitination activity. The Y223F mutant, however, showed wild-type levels of activity (Fig. 4A). The three mutationally sensitive residues are also the most highly conserved among the OTU domains, further supporting their functional importance for ubiquitin recognition. A mapping of the mutational results, color-coded by the severity of the effect of the mutation on YOD1 *in vitro* activity, is shown in Fig. 4B and highlights the importance of YOD1 interaction with the COOH-terminal ubiquitin tail for substrate recognition by YOD1 and probably other OTU domain proteins.

Task 3—Identification of in vivo partners and activity of YOD1.

During the funding period, the goals of Aim 3 (Task 3 A-C) have been accomplished. Cdc48 was identified as an *in vivo* binding partner of YOD1 (Tasks 3A and 3B). We examined the effect this interaction has on YOD1 activity (Task 3C).

Substrate Binding by YOD1

To determine if YOD1 preferentially bound polyubiquitin chains over monoubiquitin and if Cdc48 or other cellular cofactors modulated this ubiquitin binding activity, binding studies were carried using recombinant YOD1 to mono-Ub and Ub^4 analog resins in the presence or absence of ΔYOD1 yeast lysate. Each resin was incubated with ΔYOD1 lysate, recombinant YOD1 protein (~20-fold excess to endogenous YOD1 levels), or both. These experiments show that the YOD1 protein does not bind to control or monoubiquitin resins under any experimental conditions (Fig. 5A, lanes 2-7). However, YOD1 protein binds efficiently to 11-, 29-, 48-, and 63-Ub4 analog resins, whether ΔYOD1 lysate is present or not (see arrow labeled YOD1 in Fig. 5A, lanes 8-19). These results demonstrate that YOD1 binds directly to polyubiquitin chains and that this binding can occur in the absence of additional cellular cofactors.

Despite the lack of a requirement for other cellular proteins to facilitate the binding of YOD1 protein to Ub4 analog resins, a lysate protein band (migrating at an apparent molecular mass of 110 kDa) was strongly enriched in the bound fractions containing YOD1 (see arrow in Fig. 5A). Significantly, this band did not appear in bound fractions when YOD1 protein was not present, suggesting that this band represents a protein recruited to the YOD1-Ub4 complex. To establish the identity of this protein band, it was excised from the gel, trypsin-digested, and subjected to MALDI-TOF analysis. Of 56 peptides detected, 18 were a match to Cdc48 using Profound (Genomic Solutions, Ann Arbor, MI). Based on the 22% sequence coverage and a high Z-score of 2.38 (confidence level of >95%), the protein band was confidently identified as Cdc48, an essential AAA ATPase involved in numerous cellular functions ranging from chaperoning polyubiquitinated proteins to the proteasome to regulating membrane fusion and previously reported to bind YOD1.

It has been previously shown that YOD1 also cleaves polyubiquitin and that a 4-fold molar excess of enzyme cleaved Lys48-linked chains somewhat more efficiently than Lys63-linked chains. Chains of three or fewer ubiquitins were not cleaved at all. We extended these studies using stoichiometric amounts of YOD1 incubated with Lys29, Lys48, or Lys63-linked Ub4, and the reaction was analyzed for deubiquitination activity with anti-ubiquitin immunoblotting. This analysis demonstrates that YOD1 has a clear preference for Lys48-linked Ub4, although it has some activity on K63-linked Ub4 as well (Fig. 5D). Lys29-linked chains were not cleaved at all. It is notable that the extent of chain cleavage is very limited. It appears that single cleavage events are detected, with the majority of cleavages occurring in the middle of the tetraubiquitin chain. This suggests that the enzyme may be unable to efficiently release product and brings up the possibility that the action of the Cdc48 chaperone is necessary to release bound products. Alternatively, the enzyme may prefer to cleave in the middle of a chain releasing diubiquitin.

Cdc48 (in higher eukaryotes termed p97 or VCP for valosin-containing protein) was originally identified as an AAA ATPase that is involved in cell cycle regulation in yeast. Cdc48 contains two Walker ATPase domains. Published data suggests that ATP hydrolysis by Cdc48 allows the removal of ubiquitinated ER proteins from the membrane, but the exact mechanism is unclear. Cdc48 has been dubbed a segregase because of its ability to separate polypeptides from a relatively large immobile subcellular structure. The function of Cdc48 is important in a number of different biological processes including regulation of transcription, nuclear envelope formation, DNA replication, membrane fusion, endocytic trafficking, and autophagosome formation. The functional diversity of Cdc48 and the sheer number of substrates to which Cdc48 binds made identifying specific substrates of YOD1 difficult.

To determine if the interaction between YOD1 and Cdc48 was direct, we immobilized purified YOD1 on an affinity support and incubated it with His6-tagged Cdc48 purified to ~80% homogeneity from *E. coli* lysates and analyzed by SDS-PAGE and immunoblotting. Analysis demonstrates that recombinant Cdc48 protein binds to the immobilized YOD1 in an ATP-independent fashion but not to the control resin (Data not shown). These results confirm that YOD1 and Cdc48 associate through a direct interaction and that both free YOD1 and the YOD1-Cdc48 oligomeric complex bind polyubiquitin chains.

To understand how YOD1 interacts with Cdc48, I focused on UBX, the N-terminal 86 amino acid portion of YOD1. UBX is an ~80-amino acid domain where the secondary structure of the domain closely mimics the ubiquitin fold, but the primary sequence has minimal homology to ubiquitin. Other yeast proteins, such as Npl4 and Shp1, are known to interact with Cdc48 through UBX domains. To test if the UBX domain of YOD1 bound to polyubiquitin is involved in binding to Cdc48, we repeated ubiquitin-analog binding experiments using ΔYOD1 yeast lysate with recombinant full-length YOD1 and a truncated YOD1 construct lacking the N-terminal 100-residue UBX region (YOD1ΔUBX). These studies reveal that although YOD1ΔUBX bound to the Ub4 analog resins indistinguishably from the intact YOD1 protein, the protein band corresponding to the Cdc48 protein (Fig. 5B, arrows) was noticeably absent in the presence of the YOD1ΔUBX construct. Based on this observation, we conclude that the UBX region of YOD1 plays an important role in Cdc48 interaction.

Next, I investigated whether Cdc48 association with YOD1 has an effect on the deubiquitination activity of YOD1. The ability of the Cdc48-YOD1 complex to cleave Ub-AMC was analyzed and found to be only slightly slower than that catalyzed by YOD1 alone (Fig. 5C). This result shows that the association of Cdc48 with YOD1 has little effect on hydrolyzing ubiquitin-AMC, although this experiment does not eliminate the possibility that other proteins that interact with YOD1 or Cdc48 might alter the deubiquitination activity of YOD1 in a Cdc48-dependent or independent way. Together, these results show that YOD1 contains modest DUB activity but little affinity for monoubiquitin.

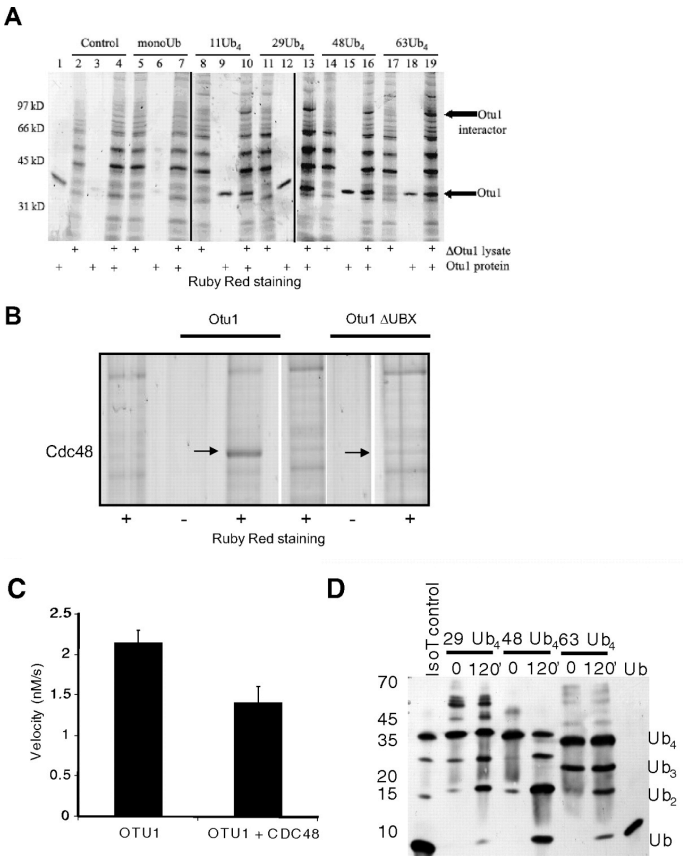

Figure 5 Protein-protein interactions of YOD1. A, ubiquitin binding activity of YOD1. YOD1 binding to ubiquitin and Ub4 resins in the presence or absence of ΔYOD1 yeast lysate is analyzed by SDS-polyacrylamide gel stained with Sypro Ruby Red. The properties of the resin are indicated above the gel, and the presence or absence of YOD1 protein and ΔYOD1 yeast lysate is indicated below the gel. B, requirement of the YOD1 N-terminus for Cdc48 association. Cdc48 association with resins containing monoubiquitin or Lys48-linked Ub4 in the presence or absence of YOD1 and YOD1ΔUBX is analyzed by a SDS-polyacrylamide gel and stained with Sypro Ruby Red. The YOD1 load lanes are 4% of total YOD1 input, and the remaining lanes are all elution fractions from the resins. The arrows indicate the YOD1 interactor, Cdc48. C, YOD1 catalytic activity in the presence of Cdc48. Activity of YOD1 is measured in the presence or absence of a 2-fold molar excess of the Cdc48 hexamer. D, YOD1 cleavage of native polyubiquitin chains. YOD1-mediated cleavage of Lys29-, Lys48-, or Lys63-linked Ub4 chains is analyzed by SDS-polyacrylamide gel and Western blotting with a monoclonal ubiquitin antibody and visualized by ECL.

KEY RESEARCH ACCOMPLISHMENTS

- Expression and purification of YOD1 (amino acids 87-262).
- Creation of a covalent complex between YOD1 and ubiquitin-Br3.
- Crystallization and structure determination of YOD1-ubiquitin complex in two crystal forms.
- Enzymatic characterization of YOD1. Biochemical analysis of mutants at the Otu domain:ubiquitin interface.
- Identification of Cdc48 as a YOD1 binding partner and characterization of YOD1 activity when in a complex.

REPORTABLE OUTCOMES

- A paper describing the determination of the crystal structure of YOD1 bound to ubiquitin and the biochemical activity has been published.

Messick TE, Russell NS, Iwata AJ, Sarachan KL, Shiekhattar R, Shanks JR, Reyes-Turcu FE, Wilkinson KD, Marmorstein R. (2008) Structural basis for ubiquitin recognition by the Otu1 ovarian tumor domain protein. J Biol Chem. 2008 Apr 18;283(16):11038-49. PMID 18270205.

- I attended the Ubiquitin Family meeting at Cold Spring Harbor Laboratory and presented the structure and activity of the YOD1.

CONCLUSIONS

In conclusion, the high-resolution structure of the Ovarian Tumor (OTU) domain in complex with ubiquitin is providing important molecular insights into the specificity and catalytic mechanism of this OTU-containing deubiquitination enzymes. Conjugation of ubiquitin can drastically alter the activity of target proteins in a number of different ways: intracellular localization, degradation, recruitment of other proteins, and alteration in enzymatic activity. Thus, ubiquitin conjugates must be tightly regulated by both ubiquitin ligases and deubiquitination enzymes. If hOTU proves to be an important player in counterbalancing the ubiquitin-conjugating activity of BRCA1, inhibiting hOTU using medicinal inhibitors may help compensate for loss of function mutations of BRCA1. These studies are providing insights into the structure and function of this new class of deubiquitination enzymes and, by extension, provide insights into BRCA1-related breast and ovarian cancer pathogenesis.

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APPENDICES

None.